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(54) **Biologically active substance-secreting hybrid gel.**

(57) The present invention provides biologically active substance-secreting hybrid gel, which consists of a biopolymeric gel and cells containing an expression vector with gene encoding biologically active substance and produce the substance.

According to the present invention, it is possible to develop a gene therapy by skin transplantation allowing stable drug medication for a long time; alleviating pains of the patients; and allowing fine adjustment of the dosage and control of genes externally without using retrovirus-derived vector that tend to invoke the risk of mutation to wild types as in the conventional prescription.

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Field of the Invention

The present invention relates to biologically active substance-secreting hybrid gel. More particularly, the present invention relates to a new hybrid gel which secrets biolgically active substance, and is useful as external-use prescriptions such as artificial skin used in the treatment of various difficult-to-cure diseases requiring long-term and continuous medication of physiological active substances in order to maintain biological functions.

Prior Art

Three methods are available to cure diseases which are caused by the loss or decrease in the functions of human cells for any reason. Namely, the lost or decreased functions are compensated for by

1) drug medication,
2) transplantation of organs, tissues or cells, or
3) gene therapy

Insulin dependent diabetes mellitus, for example, are caused by the destruction of the β-cells which produce insulin having the function of adjusting the blood sugar level in the negative direction and which occur in the islets of Langerhans of the pancreas. Patients of the insulin dependent diabetes mellitus have a high blood sugar level, and as a result, the concentration of sugar in their urine increases. When the blood sugar level remains high, the functions of various human cells are damaged, causing serious complications.

It is thus necessary to externally dispense insulin and control the blood sugar level in order to cure the insulin dependent diabetes mellitus patients. Insulin dependent diabetics must have insulin dosed several times every day for life. This is a serious physical and mental agony to the patient, and in addition, self-medication always involve risk of life because of possible mis-dispensing.

One of alternative methods to self-medication of insulin is transplantation of the pancreas or the islets of Langerhans (K. Kubota and Y. Idezuki, Nippon Rinsho: in Japanese, 48:1052, 1990). However, this treatment has a number of problems, such as, for example, few donors, difficulty of controlling immunorejection caused by the transplanted pancreas or the tissues, complicated surgical operations for transplantation requiring techniques of a high level, and hazards involved in the operation.

Gene therapy is one of the most exciting medical technique to solve the above problems, and various gene therapeis are clinically tested for treating pacients of serious deseases in the United States and other countries in the 1990s (N. M. Summers, Biotechnology 12:42, 1994). A method of treating diabetes based on the above technique has been proposed (R. F. Selden et al, The New England Journal of Medicine, 317(17): 1067, 1987). In this particular method, insulin gene is introduced into culture cells, and the cells are transplanted to the body of the patient in order to assure continuous secretion of insulin produced by the introduced gene. This method has a number of problems such as, for example, difficulty of controlling secretion of insulin from the transplanted insulin producing cells and inability of removing the transplanted cells later from the body. It is generally known that the gene therapy is a promising and advanced medical technique for not only insulin dependent diabetes mellitus and genetic diseases such as serious immune deficiency diseases but also cancer, AIDS, and other hard-to-cure diseases. For this reason, many approaches have been proposed and the gene therapy is actually conducted in practical clinical cases. Most of these gene therapy use retrovirus-derived vectors to introduce genes to the cells utilizing cell infection of the virus.

This technique to use retrovirus-derived vectors has the defect that the effectiveness of gene introduction depends on the affinity of the virus with cells, and there is a possibility that the deactivated virus vectors transform into wild retroviruses. In addition, conventional gene therapy generally have a problem of difficulty of controlling the introduced genes externally.

Summary of the Invention

The present invention intends to provide a new art of transplanting cells containing a gene which code biologically active substance into the skin and controlling the exprssion of the gene externally. More specifically, the present invention intends to solve the problems of the prior art by transplanting biologically active substance-producing cells to the skin of a human body as hybrid gel (cell-incorporating gel).

The present invention provides biologically active substance-secreting hybrid gel, which consists of biologically active substance-producing cells and biopolymeric gel.

In the case of said hybrid gel, a preferable embodiment is that the biologically active substance-producing cells are enclosed in or laminated on the biopolymeric gel, or laminated on the biopolymeric gel enclosing the biologically active substance-producing cells.

The present invention also proides biologically active substance-secreting hybrid gel, which consists of biologically active substance-producing cells, animal skin cells and biopolymeric gel.

In the case of said hybrid gel, a preferable embodiment is that the animal skin cells are laminated on the biopolymeric gel enclosing the biologically active substance-producing cells; the biologically active substance-produing cells are lainated on the biopolmeric gel enclosing the animal skin cells; the animal skin cells and the biologically active substance-producing cells are laminated on the biopolymeric gel; or the animal skin cells or the biologically active substance-producing cells are laminated on the biopolymeric gel enclosing the animal skin cells and the biologically active substance-producing cells.

Furthermore, in the case of the biologically active substance-producing cells being enclosed in the biopolymeric gel, said cells are enclosed together with meshy material or porous memrane.

In the present invention, moreover, the biologically active ubstance-producing clls may be skin cells (i.e., skin fibroblasts or skin eoidermal c1ls) which contains an expression vector recombnant with a DNA sequence encoding the biologically active substance such as insulin. And, said expresson vector may be plasmid vector pBMG-neo-ins possessing insulin cDNA and neomycin resistance gene, or plasmid vector pRIS-proins-Ifur-Ilfur-B10D which possesses mutant insulin gene expressing stable insulin by the action of furin.

According to the present invention, it will be possible to develop a gene therapy by skin transplantation allowing stable drug medication for a long time ; alleviating pains of the patients; and allowing fine adjustment of the dosage and control of gene externally without using retrovirus-derived vectors that tend to invoke the risk of mutation to wild types as in the conventional technique, is provided.

Detailed Description of the Invention

The cells of being enclosed in or laminated on the bioolymeric gel of the present invention produce a biologically active substance necessary for or deficient in the body, and the substance is continually secreted into the body. The production of biologically active substance is increased when meshy material or porous membrace, etc. are enclosed in the gel together with the biologically active substance producing cells. Thus the hybrid gel of the present invention can be effectively used as, for example, an external-use prescription such as artificial skin. The gene expressing biologically active substance is introduced to the cells by, for example, plasmid vector, and thus, unlike conventional gene therapy, no risk of conversion into wild retroviruses owing to the retrovirus-derived vectors is involved. In addition, the introduced gene can be easily controlled externally because the gene-containing cells are transplanted to the skin.

The following functions are available in concrete:

1) After transplantation, the biologically active subustance is dispensed for a long time stably without the knowing of the patient. This dramatically reduces the physical and mental agony of the patient subject to repeated medication in the conventional treatment.

2) A very simple surgery is used to transplant or remove the hybrid gel of the present invention from the skin. For this reason, the quantity of artificial skin to be transplanted can be adjusted at any time freely while watching the process condition of the treatment. It is thus easy to determine optimum conditions for treatment.

3) The rate of substance secretion from the cells in or on the gel can be controlled by means of inducible promotrs to drive a DNA sequence encoding a biologically active substance and various induction stimuli (hormones, heavy metals, temperature, etc.) applied to the transplanted artificial skin. This allows fine adjustment of the substance secretion rate.

4) The transplanted cells are enclosed in or on the gel and thus are hardly affected by immunorejection of the patient. It is thus possible to decrease the quantity of immunosuppressants generally used in the transplantation of tissues in the conventional technique. The risk of side effects owing to the use of immunosuppressants is thus greatly reduced. Of course there is no problem of immunorejection if the cells of the patient himself are used in the gene therapy because such is a self-transplantation.

5) Simple operation without the need of the patient's hospitalization is safe and free from the risk incurred in conventional treatment. Because this is the transplantation to the skin, the condition of transplantation is visible externally at all times. The transplanted artificial skin can be removed when necessary.

Various biologically active substance-producing cells can be used in the present invention for incorporating expression vector with gene therefor in the cells. For example, insulin-producing cells can be prepared by transfecting plasmid vector pBMG-neo-ins possessing cDNA of insulin and neomycin resistance gene (selection marker) to animal cells using a known method. Another method is to transfect plasmid vector pRIS-proins-Ifur-Ilfur-B10D into animal cells. This plasmid vector contains mutant insulin gene that convert proinsulin expressed from the gene into insulin by the action of furin and by the substitution of the 10th amino acid in the insulin chain B.

The gel to accommodate the biologically active substance-producing cells may be prepared from, for example, collagen, fibrin, agalose, etc. by using known methods. For example, the hybrid gel containing cells with insulin genes therein may be prepared and used as artificial skin for curing diabetics in the following manner:

(1) Pieces of skin of experimental animal are collected. Epidermal cells and fibroblasts, two major constituent cells of skin, are separated from skin and cultured.
(2) Expression vector containing insulin gene is transdced into these cells to derive insulin-secreting cell lines.
(3) Hybrid type artificial skin with the insulin-secreting function is constructed from these cell lines using collagen gels, etc.
(4) The insulin-secreting hybrid type artificial skin is transplanted.

To be more specific, the hybrid gel secreting biologically active substance of the present invention can be manufactured in accordance with the method of Asaga et al (H. Asaga et al, Experimental Cell Research, 193: 167, 1991) as follows:

Quadruple concentrated medium of cell culture, serum, purified water, and, for example, collagen (0.5% solution) are mixed in the ratio of 2.5:1:2.5:4 according to the required quantity while cooling the mixture with ice. The aqueous solution of 1N sodium hydroxide is drip-mixed in the mixture to adjust to pH 7.4. The mixture is separately injected into hydrophobic plastic laboratory dishes of 35 mm in diameter, 2 ml in each dish. The dishes are immediately transferred to a 37°C thermostat. The collagen solidifies in several minutes to produce gel. Biologically active substance-producing cells are mixed into the above mixture just before collagen solidifies in order to enclose the cells in the gel.

To allow meshy material or porous membrane to coexist in the gel, one needs only to mix these in the above solution together with the biologically active substance-producing cells.

Commercially available culture solutions, serum and collagen can be used in the present invention.

It is effective to give an appropriate strength to the collagen gels to facilitate transplantation of the product to the skin. An appropriate strength can be given to the gel by, for example, mixing an appropriate number of skin-derived fibroblasts according to the method of Bell et al (E. Bell et al, Proceedings of the National Academy of Sciences, 76(3): 1274, 1979). An appropriate strength can be given to the gel as a result of contraction of the gel owing to the fibroblasts. Skin-derived fibroblasts can be obtained, for example, by culturing a small portion of skin collected from the patient according to the primary explant technique (R.I. Freshrey, Culture of Animal Cells, Alan R. Liss, Inc., New York, 1987).

It is also effective to make the gel surface active to ensure good attachment to the skin by overlaying by culture skin-derived epidermal cells on the gel before they are transplanted to the skin.

Skin-derived epidermal cells to be overlaid on the gel may be obtained by culturing epidermal cells obtained from the skin of the patient himself in the same way as described for the fibroblasts using the method of, for example, Green et al (H. Green et al, Proceedings of the National Academy of Sciences 76: 5665, 1978).

It goes without saying that the present invention is effective also when the gel is transplanted subcutaneously without overlaying epidermal cells.

Practically, many forms are available.

Examples

Examples are shown below to further describe the present invention in detail. These examples should not be construed as limiting.

Example 1

Hybrid gel (or simply Gel hereafter) of the present invention were prepared to evaluate the method of medication and their application to the treatment of diabetics by conducting in-vitro experiment and in-vivo experiment with model animals of diabetic as described below.

In-vitro Experiment

Gel containing proinsulin-producing cells were cultured, and proinsulins secreted into the culture medium were measured.

1) Materials

Three types of skin-derived cell lines were used.

(1) Mouse embryo fibroblasts (NIH3T3)

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Field of the Invention

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It is thus necessary to externally dispense insulin and control the blood sugar level in order to cure the insulin dependent diabetes mellitus patients. Insulin dependent diabetics must have insulin dosed several times every day for life. This is a serious physical and mental agony to the patient, and in addition, self-medication always involve risk of life because of possible mis-dispensing.

One of alternative methods to self-medication of insulin is transplantation of the pancreas or the islets of Langerhans (K. Kubota and Y. Idezuki, Nippon Rinsho: in Japanese, 48:1052, 1990). However, this treatment has a number of problems, such as, for example, few donors, difficulty of controlling immunorejection caused by the transplanted pancreas or the tissues, complicated surgical operations for transplantation requiring techniques of a high level, and hazards involved in the operation.

Gene therapy is one of the most exciting medical technique to solve the above problems, and various gene therapeis are clinically tested for treating pacients of serious deseases in the United States and other countries in the 1990s (N. M. Summers, Biotechnology 12:42, 1994). A method of treating diabetes based on the above technique has been proposed (R. F. Selden et al, The New England Journal of Medicine, 317(17): 1067, 1987). In this particular method, insulin gene is introduced into culture cells, and the cells are transplanted to the body of the patient in order to assure continuous secretion of insulin produced by the introduced gene. This method has a number of problems such as, for example, difficulty of controlling secretion of insulin from the transplanted insulin producing cells and inability of removing the transplanted cells later from the body. It is generally known that the gene therapy is a promising and advanced medical technique for not only insulin dependent diabetes mellitus and genetic diseases such as serious immune deficiency diseases but also cancer, AIDS, and other hard-to-cure diseases. For this reason, many approaches have been proposed and the gene therapy is actually conducted in practical clinical cases. Most of these gene therapy use retrovirus-derived vectors to introduce genes to the cells utilizing cell infection of the virus.

This technique to use retrovirus-derived vectors has the defect that the effectiveness of gene introduction depends on the affinity of the virus with cells, and there is a possibility that the deactivated virus vectors transform into wild retroviruses. In addition, conventional gene therapy generally have a problem of difficulty of controlling the introduced genes externally.

Summary of the Invention

The present invention intends to provide a new art of transplanting cells containing a gene which code biologically active substance into the skin and controlling the exprssion of the gene externally. More specifically, the present invention intends to solve the problems of the prior art by transplanting biologically active substance-producing cells to the skin of a human body as hybrid gel (cell-incorporating gel).

The present invention provides biologically active substance-secreting hybrid gel, which consists of bilogically active substance-producing cells and biopolymeric gel.

In the case of said hybrid gel, a preferable embodiment is that the biologically active substance-producing cells are enclosed in or laminated on the biopolymeric gel, or laminated on the biopolymeric gel enclosing the biologically active substance-producing cells.

The present invention also proides biologically active substance-secreting hybrid gel, which consists of biologically active substance-producing cells, animal skin cells and biopolymeric gel.

In the case of said hybrid gel, a preferable embodiment is that the animal skin cells are laminated on the biopolymeric gel enclosing the biologically active substance-producing cells; the biologically active substance-produing cells are lainated on the biopolmeric gel enclosing the animal skin cells; the animal skin cells and the biologically active substance-producing cells are laminated on the biopolymeric gel; or the animal skin cells or the biologically active substance-producing cells are laminated on the biopolymeric gel enclosing the animal skin cells and the biologically active substance-producing cells.

Furthermore, in the case of the biologically active substance-producing cells being enclosed in the biopolymeric gel, said cells are enclosed together with meshy material or porous memrane.

In the present invention, moreover, the biologically active ubstance-producing clls may be skin cells (i.e., skin fibroblasts or skin eoidermal c1ls) which contains an expression vector recombnant with a DNA sequence encoding the biologically active substance such as insulin. And, said expresson vector may be plasmid vector pBMG-neo-ins possessing insulin cDNA and neomycin resistance gene, or plasmid vector pRIS-proins-Ifur-IIfur-B10D which possesses mutant insulin gene expressing stable insulin by the action of furin.

According to the present invention, it will be possible to develop a gene therapy by skin transplantation allowing stable drug medication for a long time ; alleviating pains of the patients; and allowing fine adjustment of the dosage and control of gene externally without using retrovirus-derived vectors that tend to invoke the risk of mutation to wild types as in the conventional technique, is provided.

Detailed Description of the Invention

The cells of being enclosed in or laminated on the bioolymeric gel of the present invention produce a biologically active substance necessary for or deficient in the body, and the substance is continually secreted into the body. The production of biologically active substance is increased when meshy material or porous membrace, etc. are enclosed in the gel together with the biologically active substance producing cells. Thus the hybrid gel of the present invention can be effectively used as, for example, an external-use prescription such as artificial skin. The gene expressing biologically active substance is introduced to the cells by, for example, plasmid vector, and thus, unlike conventional gene therapy, no risk of conversion into wild retroviruses owing to the retrovirus-derived vectors is involved. In addition, the introduced gene can be easily controlled externally because the gene-containing cells are transplanted to the skin.

The following functions are available in concrete:

1) After transplantation, the biologically active subustance is dispensed for a long time stably without the knowing of the patient. This dramatically reduces the physical and mental agony of the patient subject to repeated medication in the conventional treatment.

2) A very simple surgery is used to transplant or remove the hybrid gel of the present invention from the skin. For this reason, the quantity of artificial skin to be transplanted can be adjusted at any time freely while watching the process condition of the treatment. It is thus easy to determine optimum conditions for treatment.

3) The rate of substance secretion from the cells in or on the gel can be controlled by means of inducible promotrs to drive a DNA sequence encoding a biologically active substance and various induction stimuli (hormones, heavy metals, temperature, etc.) applied to the transplanted artificial skin. This allows fine adjustment of the substance secretion rate.

4) The transplanted cells are enclosed in or on the gel and thus are hardly affected by immunorejection of the patient. It is thus possible to decrease the quantity of immunosuppressants generally used in the transplantation of tissues in the conventional technique. The risk of side effects owing to the use of immunosuppressants is thus greatly reduced. Of course there is no problem of immunorejection if the cells of the patient himself are used in the gene therapy because such is a self-transplantation.

5) Simple operation without the need of the patient's hospitalization is safe and free from the risk incurred in conventional treatment. Because this is the transplantation to the skin, the condition of transplantation is visible externally at all times. The transplanted artificial skin can be removed when necessary.

Various biologically active substance-producing cells can be used in the present invention for incorporating expression vector with gene therefor in the cells. For example, insulin-producing cells can be prepared by transfecting plasmid vector pBMG-neo-ins possessing cDNA of insulin and neomycin resistance gene (selection marker) to animal cells using a known method. Another method is to transfect plasmid vector pRIS-proins-Ifur-IIfur-B10D into animal cells. This plasmid vector contains mutant insulin gene that convert proinsulin expressed from the gene into insulin by the action of furin and by the substitution of the 10th amino acid in the insulin chain B.

The gel to accommodate the biologically active substance-producing cells may be prepared from, for example, collagen, fibrin, agalose, etc. by using known methods. For example, the hybrid gel containing cells with insulin genes therein may be prepared and used as artificial skin for curing diabetics in the following manner:

(1) Pieces of skin of experimental animal are collected. Epidermal cells and fibroblasts, two major constituent cells of skin, are separated from skin and cultured.
(2) Expression vector containing insulin gene is transdced into these cells to derive insulin-secreting cell lines.
(3) Hybrid type artificial skin with the insulin-secreting function is constructed from these cell lines using collagen gels, etc.
(4) The insulin-secreting hybrid type artificial skin is transplanted.

To be more specific, the hybrid gel secreting biologically active substance of the present invention can be manufactured in accordance with the method of Asaga et al (H. Asaga et al, Experimental Cell Research, 193: 167, 1991) as follows:

Quadruple concentrated medium of cell culture, serum, purified water, and, for example, collagen (0.5% solution) are mixed in the ratio of 2.5:1:2.5:4 according to the required quantity while cooling the mixture with ice. The aqueous solution of 1N sodium hydroxide is drip-mixed in the mixture to adjust to pH 7.4. The mixture is separately injected into hydrophobic plastic laboratory dishes of 35 mm in diameter, 2 ml in each dish. The dishes are immediately transferred to a 37°C thermostat. The collagen solidifies in several minutes to produce gel. Biologically active substance-producing cells are mixed into the above mixture just before collagen solidifies in order to enclose the cells in the gel.

To allow meshy material or porous membrane to coexist in the gel, one needs only to mix these in the above solution together with the biologically active substance-producing cells.

Commercially available culture solutions, serum and collagen can be used in the present invention.

It is effective to give an appropriate strength to the collagen gels to facilitate transplantation of the product to the skin. An appropriate strength can be given to the gel by, for example, mixing an appropriate number of skin-derived fibroblasts according to the method of Bell et al (E. Bell et al, Proceedings of the National Academy of Sciences, 76(3): 1274, 1979). An appropriate strength can be given to the gel as a result of contraction of the gel owing to the fibroblasts. Skin-derived fibroblasts can be obtained, for example, by culturing a small portion of skin collected from the patient according to the primary explant technique (R.I. Freshrey, Culture of Animal Cells, Alan R. Liss, Inc., New York, 1987).

It is also effective to make the gel surface active to ensure good attachment to the skin by overlaying by culture skin-derived epidermal cells on the gel before they are transplanted to the skin.

Skin-derived epidermal cells to be overlaid on the gel may be obtained by culturing epidermal cells obtained from the skin of the patient himself in the same way as described for the fibroblasts using the method of, for example, Green et al (H. Green et al, Proceedings of the National Academy of Sciences 76: 5665, 1978).

It goes without saying that the present invention is effective also when the gel is transplanted subcutaneously without overlaying epidermal cells.

Practically, many forms are available.

Examples

Examples are shown below to further describe the present invention in detail. These examples should not be construed as limiting.

Example 1

Hybrid gel (or simply Gel hereafter) of the present invention were prepared to evaluate the method of medication and their application to the treatment of diabetics by conducting in-vitro experiment and in-vivo experiment with model animals of diabetic as described below.

In-vitro Experiment

Gel containing proinsulin-producing cells were cultured, and proinsulins secreted into the culture medium were measured.

1) Materials

Three types of skin-derived cell lines were used.

(1) Mouse embryo fibroblasts (NIH3T3)

(2) Rat skin fibroblasts containing insulin gene (RSFins)

(3) Rat skin epidermal cells containing insulin gene (RSKins)

RSFins and RSKins were prepared by transduction of insulin gene (G. I. Bell et al, Nature, 284 (6):26, ;1980) into fibroblasts (RSF) and epidermal cells (RFK), respectively, which were obtained from the primary culture of rat skin. Insulin gene was transfected into the fibroblasts and epidermal cells using plasmid vector pBMG-neo-ins which possesses human insulin cDNA (Y. Kawakami et al, Diabetes 41: 956, 1992) according to the method of Chen and Okayama (C. Chen and H. Okayama, Molecular and Cellular Biology 7(8): 2745, 1987). The vector-containing cells were then selectively increased in culture media of G418 of 400 μg/ml concentration.

These cells had no processing enzymes to insulin and thus secreted proinsulin, precursor of insulin. It should be noted that proinsulin also have the functions of insulin (S.N. Davis et al., Journal of Clinical Endocrinogy and Metabolism, 75 (5): 1282-1288, 1992).

2) Culture medium

The culture medium for RSFins consisted of Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY), to which fetal bovine serum (HyClone, Logan, Utah) was added to the ratio of 10% (medium A).

The culture medium for RSKins consisted of a 7:3 mixture of Dulbecco's modified Eagle's medium and MCDB152 medium (Kyokuto, Tokyo), to which hydrocortisone (0.4 μg/ml), insulin (5 μg/ml), transferrin (5 μg/ml), triiodothyronine (2 nM), cholera toxin (0.1 nM), adenine (100 μM) and fetal bovine serum (10%) were added (medium B).

The cells were enclosed in and/or laminated on the gel. The resultant hybrid gel were cultured in medium A.

3) Procedures

5×10^5 cells of RSFins each were enclosed in the gel and laminated on another gel to prepare Gels A and B, respectively. Gels C and D were further prepared by placing 5×10^5 cells of RSKins each in and on the gels, respectively. NIH3T3 cells of the same number were enclosed in Gels C and D to give contractive function. The structure of these Gels is summarized in Table 1. These cells were cultured at 37°C. One day after the preparation, 2 ml culture medium was added to continue culture. Thereafter, the culture medium was replaced with new one every other day. The retrieved culture medium was frozen for storage, melted when necessary, and measured for proinsulin concentration in the culture medium. Proinsulin concentration was measured as a value of immunoreactive insulin (IRI) using the EIA kit (Sanko Junyaku, Tokyo).

Table 1

Gel	Cells in the gel	Cells on the gel
Gel A	RSFins ($N=5\times10^5$)	None
Gel B	None	RSFins ($N=5\times10^5$)
Gel C	RSKins ($N=5\times10^5$) NIH3T3 ($N=5\times10^5$)	None
Gel D	NIH3T3 ($N=5\times10^5$)	RSKins ($N=5\times10^5$)

4) Results

The results of this experiment are shown in Table 2. Both of the cell lines enclosed in and laminated on the gel secreted a stable quantity of proinsulins into the culture medium for 25 culture days. It is thus possible to deliver proinsulins into the body when these Gels are transplanted to the skin.

Table 2

Secretion of proinsulins from Gels to culture medium (μU/ml/day)				
Gel	Days of culture			
	4 days	8 days	14 days	24 days
Gel A	252	248	340	399
Gel B	239	255	350	325
Gel C	201	215	340	363
Gel D	210	212	340	328

In-vivo Experiment 1

Proinsulin producing cell-enclosed hybrid gels were transplanted to model diabetic animals to evaluate the curing effects by measuring blood sugar level.

1) Experiment animals

200 mg/kg streptozotocin (Sigma, St.Louis, Mo) was intra-peritoneally administered to the Ba1b/c nude mice (5-weeks old, male) at three times in four days to induce the diabetic condition. The mice were used for experiments when they were 7 weeks old.

2) Materials

Three types of rat skin-derived cell lines were used.

(1) RSF
(2) RSFins
(3) RSKins

3) Method for preparing Gels

5 x 10^5 cells of RSFins were enclosed in collagen gel and RSKins cells of the same number were laminated on the surface of said gel to prerare Gel E. 5 x 10^5 cells of RSF were enclosed in collagen gel and RSKins cells of the same number were laminated on the surface of said gel to prerare Gel F. The structure of these Gels is summarized in Table 3. These cells were cultured for six days at 37°C and then used for transplantation. Gels E and F produced proinsulins 484 and 404 μ IU/day, respectively.

Table 3

Gel	Cells in the Gel	Cells on the gel
Gel E	RSFins (N=5×10^5)	RSKins (N=5×10^5)
Gel F	RSF (N=5×10^5)	RSKins (N=5×10^5)

3) Procedures

The skin of two of the above model diabetic animals was cut away in the area of approximately 25 and 200 mm square, respectively, and Gels E and F, cultured for six days after preparation, were cut and transplanted for the full naked area (cut Gel weight was 24 and 191 mg by wet weight, respectively). After transplantation, about 20 μl blood was collected from the tail of the animals (ID Nos. 3 and 4) every other day to measure the blood sugar level. Two non-transplanted diabetic animals (ID Nos. 1 and 2) were used for control. The blood sugar level was measured using glucose CII test (Wako Pure Chemical Industry, Osaka).

4) Results

The results of this experiment are shown in Table 4. The control animals show a continuous rise in the blood sugar level while such a rise in the blood sugar level is suppressed and a tendency of decreasing blood sugar level is indicated in the Gel-transplanted animals.

Table 4

Effect of Gel transplantation to diabetic mouse

Experiment group	ID No.	Blood sugar level (mg/dl) Before dosing STZ: 2 hours before dosing STZ	After dosing STZ: 10th day after dosing STZ (1 day before gel transplantation)	After dosing STZ: 5th day after gel trans-plantation
Control group	1	108	380	557
	2	113	415	511
Gel trans-planted group	3	90	489	437
	4	88	392	331

Note: STZ = Streptozotocin

In-vivo Experiment 2

Proinsulin producing cell-enclosed hybrid gels were transplanted to model diabetic animals to evaluate the curing effects by measuring blood sugar lebel and body weight of the animals.

1) Experimental animals

200 mg/kg of streptozotocin (Sigma) was intraperitoneally administered to the Bulb/c nude mice (7-weeks old, male) at each two days to induce the diabetic condition. The transplantation of hybrid gel was conducted after two days of the administration of streptozotocin.

2) Materials

Three types of rat skin-derived cell lines were used.

(1) RSF

(2) RSFins

(3) RSK

3) Method for preparing Gels

10^6 cells of RSFins were enclosed in collagen gel and RSK cells of the same number were laminated on the surface of said gel to prepare Gel M. 10^6 cells of RSK were enclosed in collagen gel and RSFins cells of the same number were laminated on said gel to prepare Gel N. The structure of these Gels is summarized in Table 5. These cells were cultured for 7 days at 37°C and then used for transplantation. Gels M and N produced proinslin 300.8 and 1.5μ IU/hour, respectively.

Table 5

Gel	Cells in the gel	Cells on the gel
Gel M	RSFins (N=10^6)	RSK (N=10^6)
Gel N	RSF (N=10^6)	RSK (N=10^6)

4) Procedures

The skin at right side abdomen of three animals was cut away in the form of a circle of 8-10mm diameter, and Gel M, cultured for 7 days after preparation was transplanted for the naked area. After tranplan-

tation, about 5 μl blood was collected from the tail of the animal every other day to measure the blood sugar level. The body weight of the animals were also measured evry other day. Remaining three animals to which Gel N was transplanted in a same manner were used for control. The blood sugar level was measured using Gultest-E (Sanwa Chemical Institute, Nagoya, Japan).

5) Results

The results of this experiment were shown in Table 6. The Gel N-transplanted animals show a continuous rise in the blood sugar level while such a rise in level is suppressd in the Gel M-transplanted group. The Gel M group showed an inhibitory effect on decrease of body weight as shown in the Gel N group in a course of experiment.

Table 6

Effects of Gel transpalntaion to diabetic mouse

	Body Height (B.W: upper: g) and Blood Sugar Level (B.S.L: lower: mg/dl)										
Days before/after transplantation	-3	-1	3	5	7	9	11	13	15	17	19
Gel N-Group (control)	24.7 92	23.3 251	18.2 258	17.9 315	14.8 394	17.9 >500	17.7 >500	17.0 >500	15.9 >500	15.7 384	15.7 >500
	21.0 116	20.2 224	16.9 193	17.2 337	14.3 320	17.0 453	16.6 340	16.6 >500	15.8 >500	16.8 494	16.9 423
	22.1 97	20.4 268	15.4 222	14.8 274	12.8 >500	14.6 >500	13.5 330	13.5 >500	13.2 >500	12.1 >500	NT NT
Ave. of B.W. = Ave. of B.S.L =	22.6 102	21.3 248	16.8 224	16.6 309	14.0 >405	16.5 >484	15.9 >390	15.7 >500	15.0 >500	14.9 >459	16.3 >462
Gel M-Group	22.2 140	20.8 217	17.4 109	17.5 290	17.1 471	17.5 >500	18.1 389	18.2 372	18.8 >500	17.6 388	18.4 >500
	22.3 97	21.8 206	17.7 172	17.1 295	18.5 415	18.4 442	19.3 345	19.9 403	20.6 >500	20.6 413	20.6 401
	20.7 127	20.2 296	18.6 137	18.5 197	16.8 335	16.5 423	17.5 314	18.1 369	18.6 443	19.0 >500	18.4 443
Ave. of B.W. = Ave. of B.S.L =	21.7 121	20.9 240	17.9 139	17.7 261	17.5 407	17.5 >455	18.3 349	18.7 381	19.3 >481	19.1 >434	19.1 >448

Note) TN indicates non-testable cases because of the death of subject.

Example 2

Another form of hybrid gel of the present invention was prepared and the effects were evaluated using in-vitro experiment and in-vivo experiment on diabetic-model animals as described below.

In-vitro Experiment 1

This experment was performed by culturing the hybrid gel containing insulin-producing cells, to which mu-

tant insulin gene encoding proinsulin susceptible to furin was introduced, and measured levels of IRI secreted into the culture medium.

1) Materials

Two types of skin-derived cell lines were used.

(1) Rat skin fibroblasts with mutant insulin gene being convertible with furin (RSFinsfur).

(2) RSK

RSFinsfur were prepared by introducing mutant insulin gene (D. J. Groskreutz et al, The Journal of Biological Chemistry, 269 (8), 6241, 1994), capable of processing by furin (insulins occur when the proinsulin chains are cleaved as two portions), to the fibroblasts (RSF) obtained from rat skin by primary culture. The gene was transducted into RSF using plasmid expressiom vectors pRIS-proins-Ifur-IIfur-B10D which contained the above-mentioned mutant insulin gene, according to the same method described in Example 1. The vector-transducted cells were then selectively increased in culture medium containing G418 of 600 µg/ml concentration.

Thirty-two clones were isolated from these cells and the clone of the highest IRI value were selected. This clone (RSFinsfur) secreted 24.5 µ IU/hour IRI per 10^6 cells in the culture medium.

These cells simultaneously expressed processing enzyme, furin for conversion of insulin, and thus proinsulin, precursor of insulin, was converted into insulin depending on the furin activity of the cells.

The RSFinsfur cells were immunohistologically studied using anti-furin monoclonal antibody (Genentech, South San Francisco,CA) and anti-insulin rabbit serum (Austral Biological, San Ramon, CA). The results are shown in Table 7. It was confirmed immunohistologically that these cells produce insulins and furin.

Table 7

Type of Antibody	Result of staining
Anti-furin monoclonal antibody	Positive
Anti-insulin rabbit serum	Positive

2) Culture medium

Culture media A and B used in Example 1 were used for RSFinsfur and RSK, respectively. Culture medium A was used after the cells were enclosed in and lanimated on the gel.

3) Procedures

Gel G was prepared by enclosing 3 x 10^6 cells of RSFinsfur in the gel which was then laminated RSK cells of the same number. The structure of Gel G is summarized in Table 8. Gel G was cultured at 37°C in a 6 ml culture medium. The culture medium was replaced with new one every other day. The Gel on the 8th day of culture was rinsed with culture medium three times, and the culture medium was replaced with new one. The Gel was further cultured for 8 hours. The IRI value of the culture medium was measured with the insulin EIA.

Table 8

Gel	Cells in the gel	Cells on the gel
Gel G	RSFinsfur (N=3×10^6)	RSK (N=3×10^6)

4) Results

The results of this experiment confirmed that Gel G secreted 25.2 µ IU IRI in eight hours. The Gel G secretes stable insulin for many hours, and thus, insulin can be delivered into the body when the Gel is transplanted to the skin.

In-vitro Experiment 2

This experiment was performed to examine a method to increase secretion of insulin from the hybrid gel containing insulin-producing cells, to which mutant insulin gene encoding proinsulin susceptible to furin was introduced.

1) Materials

The same cells as used in the above in-vitro experiment were used.

2) Culture medium

The same culture media as used in the above in-vitro experiment were used in the same manner.

3) Procedures

Gel H was prepared by enclosing 10^6 cells of RSFinsfur in the gel, which was then laminated with RSK cells of the same number. Gels I and J were prepared by introducing polyglycolic acid (PGA) meshes (Davis + Geck, Manati, PR) cut to a circular form of 15 cm and 25 cm in diameter, respectively, into the gels simultaneously with the enclosure of RSFinsfur cells of the same number. The gel was then laminated with RSK. The structure of these Gels is shown in Table 9.

Table 9

Gel	Cells in the gel	Material in the gel	Cells on the gel
Gel H	RSFinsfur (N=10^6)	None	RSK (N=10^6)
Gel I	RSFinsfur (N=10^6)	PGA mesh 15mm in dia.	RSK (N=10^6)
Gel J	RSFinsfur (N=10^6)	PGA mesh 25mm in dia.	RSK (N=10^6)

These Gels were cultured at 37°C in a 2 ml culture medium, respectively. Culture medium was replaced with new one every or every other day. The Gels were rinsed three times on the 8th day of culture with culture medium, and the culture medium was replaced with new one. The Gels were further cultured for 8 hours in the new medium. A small quantity (50 μl) of culture medium was sampled during the period to measure the IRI value in the culture medium with insulin EIA.

4) Results

The results of this experiment are shown in Table 10. The Gels I and J containing both meshes and cells and further laminated with RSK cells secreted a significantly greater quantity of insulin than Gel H which contained only cells in and on the gel. It is thus confirmed that the presence of mash in the gel is effective for increasing the secretion of insulin from the cells.

Table 10

Secretion of insulin from Gels to culture medium				
	Cumulative IRI value (μIU/gel)			
	1 hour	2 hours	4 hours	8 hours
Gel H (N=4)	0.8	2.4	4.1	7.5
	1.6	3.3	3.5	6.7
	1.6	2.5	3.5	5.0
	0.8	0.8	4.1	4.1
Gel I	8.4	8.4	21.9	37.1
Gel J	9.3	15.4	30.3	47.6

In-vivo Experiment

Insulin producing cell-enclosed gel was transplanted to model diabetic animals to evaluate effects of the hybrid gel transplantation by measuring the weight and blood sugar level of the animals.

1) Experiment animals

200 mg/kg of streptozotocin was intraperitoneally administered to the Ba1b/c nude mice (7-week old, male) at twice in two days to induce the high blood sugar condition. The experiment was started after confirming that the mice showed a high blood sugar levels.

2) Materials

Three types of rat skin-derived cell lines were used.

(1) RSF

(2) RSFinsfur
(3) RSK

3) Method for preparing Gels (artificial skin)

RSFinsfur cells of 3 x 10^6 were enclosed in collagen gel and RSK cells of the same number were laminated on the surface of said gel to prepare Gel K. 3 x 10^6 cells of RSF were enclosed in collagen gel and RSK cells of the same number were laminated on the surface of said gel to prepare Gel L. The structure of these Gels is summarized in Table 11.

Table 11

Gel	Cells in the gel	Cells on the gel
Gel K	RSFinsfur ($N=3\times10^6$)	RSK ($N=3\times10^6$)
Gel L	RSF ($N=3\times10^6$)	RSK ($N=3\times10^6$)

4) Procedures

The skin at the back of the model diabetic animals was cut away in the form of a circle of approximately 11 mm in diameter, and Gel K, cultured for 8 days after preparation and contracted to approximately 10 mm in diameter, was transplanted to the naked area on the animals. Gel L with cells not transduced with gene was transplanted in the same manner for control. After transplantation, the weight of the animals was measured and approximately 5 µl blood was sampled from the tail every other day to measure blood sugar level using Gultest-E.

5) Results

The results of this experiment are shown in Table 12. The control animals (Gel L transplanted group) show a decrease in the weight and increase in the blood sugar level while three was a tendency that increase in the weight and decrease in the blood sugar level were observed for Gel K (containing mutant insulin gene-transduced cells) transplanted group. This confirms improvements in the diabetic symptoms.

Table 12

Effects of Gel transplantatin to diabetic mouse

Days after transplantation	0	2	4	6	7	8
Treated group (Gel K group)	19.6 181	18.2 119	18.4 126	19.7 230	19.6 335	19.6 288
Control group (Gel L group)	19.8 152	17.5 104	18.0 112	18.0 236	18.1 231	17.8 278
	21.4 283	18.0 119	18.2 229	17.9 428	17.6 426	18.3 500

Days after transplantation	9	10	11	12	13
Treated group (Gel K group)	20.1 362	20.1 238	21.0 312	22.0 333	22.2 380
Control group (Gel L group)	18.4 318	19.0 328	19.1 286	19.5 334	19.6 468
	17.4 405	17.4 393	18.0 352	18.3 397	18.3 641

Note) Body weight: upper
Blood insulin level: lower

Claims

1. Biologically active substance-secreting hybrid gel, which consists of biologically active substance-producing cells and biopolymeric gel.

2. The hybrid gel of claim 1, wherein the biologically active substance-producing cells are enclosed in the biopolymeric gel.

3. The hybrid gel of claim 1, wherein biologically active substance-producing cells are laminated on the biopolymeric gel.

4. The hybrid gel of claim 1, wherein the biologically active substance-producing cells are laminated on the biopolymeric gel enclosing the bioloically active substance-producing cells.

5. Biologically active substance-secreting gel, which consists of biologically active substance-producing cells, animal skin cells and biopolymeric gel.

6. The hybrid gel of claim 5, wherein the animal skin cells are laminated on the biopolymeric gel enclosing the biologically active substance-producing cells.

7. The hybrid gel of claim 5, wherein the biologically active substance-producing cells are laminated on the biopolymeric gel enclosing the animal skin cells.

8. The hybrid gel of claim 5, wherein the biologically active substance-producing cells and the animal skin cells are enclosed in the biopolymeric gel.

9. The hybrid gel of claim 5, wherein the animal skin cells or the biologically active substance-producing cells are laminated on the biopolymeric gel enclosing the animal skin cells and the biologically active substance-producing cells.

10. The hybrid gel of claim 2, 4, 6, 8 or 9, wherein the biologically active substance-producing cells are enclosed in the biopolymeric gel together with meshy material or porous membrane.

11. The hybrid gel of any one of claims 5 through 9, wherein the enclosed animal skin cells are skin fibro-

blasts and the laminated animal skin cells are skin epidermal cells.

12 The hybrid gel of any one of claims 1 through 10, wherein the biologically active substance-producing cells are cells which contains an expression vector recombnant with a DNA sequence encoding a biologically active substance.

13. The hybrid gel of claim 12, wherein the iologically active substance-producing cells are skin fibroblasts or skin epidermal cells which contains an expression vector recombinant with a DNA sequence encoding insulin.

14. The hybrid gel of claim 13, wherein the expression vector is plasmid vector pBMG-neo-ins.

15. The hybrid gel of claim 13, wherein the expression vector is plasmid vector pRIS-proins-Ifur-IIfur-B10D.

16. An external-use prescription whose main component is the hybrid gel of any one of claim 1 through 15.